METHODS FOR DIAGNOSIS OF ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS

Technical field

The present invention relates to methods for the diagnosis of allergic bronchopulmonary aspergillosis (ABPA) and
5 recombinant allergens to be used in the methods. During the priority year the recombinant allergen that in the priority application was called rAsp f2 has officially been named rAsp f6. The official name is used in this text.

10 **Technical Background**

Allergic bronchopulmonary aspergillosis (ABPA). Allergic bronchopulmonary aspergillosis is the most severe allergic complication caused by Aspergillus species, mainly A. fumigatus. ABPA is the result of hypersensitivity to
15 Aspergillus-antigens mainly in patients suffering from long-standing atopic asthma (8-12) or cystic fibrosis (16-19). Although originally considered as a rare disease (13), ABPA is currently recognized with much greater frequency. ABPA with varied clinical presentations has been reported to occur
20 in about 15% of the asthmatic patients sensitized to A. fumigatus (14,15), while in patients with cystic fibrosis the reported incidence varies from 10 to 35% (16,17). ABPA has been described as an immune disease that ranges from asthma to fatal destructive lung disease with defined clinical,
25 serological, radiological and pathological features (8,18-22). Because of its severity ABPA should be ruled out in patients with chronic asthma or cystic fibrosis exhibiting immediate cutaneous reactivity to A. fumigatus (8). The diagnostic criteria for ABPA are asthma or cystic fibrosis,
30 history of roentgenographic infiltrates (in most cases), immediate cutaneous reactivity to A. fumigatus extracts, elevated total serum IgE, precipitating antibodies to A. fumigatus, peripheral blood eosinophilia, elevated specific serum IgE and IgG to A. fumigatus as compared to sera from
35 patients with asthma and cutaneous reactivity to Aspergillus, but without ABPA, and proximal (central) bronchiectasis with

normal tapering of distal bronchi (23-25). In cases where all
criteria are present, diagnosis is readily made (26).
However, all of the eight criteria are rarely present at the
same time even in classic ABPA-patients with central
5 bronchiectasis. With exception of bronchiectasis and to some
extent elevated specific serum IgE and IgG to A. fumigatus,
none of the diagnostic criteria are specific for ABPA (26).
Furthermore, pulmonary infiltrates and central bronchiectasis
are commonly detected in patients suffering from cystic
10 fibrosis also in the absence of sensitization to A.
fumigatus, which makes a diagnosis of ABPA in patients with
cystic fibrosis even more difficult (16). Therefore,
serologic identification of ABPA has a greater diagnostic
potential, but is, however, hampered by the lack of
15 standardized, reliable fungal extracts (5,7,27-29).

  Aspergillus fumigatus antigens. The major problem in the
immunodiagnosis of diseases related to A. fumigatus stems
from the antigenic compexity of the fungus. Antigen/allergen
extracts of A. fumigatus contain hundreds of different
20 proteins (6,30,31), of which a limited subset are able to
bind human serum IgE (6,32,33,35). The fungus has been
reported to produce more than 40 IgE-binding components which
generate complex IgE-binding patterns when extracts are
examined by Western blot analysis using sera from allergic
25 individuals (32,33). To make the picture even more
complicated, serum IgE from different patients recognize
highly variable patterns of fungal proteins (6,36). In the
case of patients suffering from ABPA, depending on the stage
of the disease, different allergenic "fingerprints" may be
30 obtained with serum of the same patient taken at different
times, even if fungal extract from the same batch is used
(36,37).

  It has been suggested to use purified native allergenic
components instead of crude allergen extracts for diagnosing
35 ABPA (79). Recombinant A. fumigatus allergens with
connections to ABPA have been described earlier (71,83).

The inventors are named authors in a number of articles about recombinant allergens from A. fumigatus (cloning and expression: 39,43,49,51,52,82, and diagnostic use: 59,66,32,71,76,81).

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Result of International-Type Search during the priority year.

The references 66, 79 and 84 have been categorised as being of particular relevance.

Banerjee et al., (84) describes antigens that cannot be
10 intracellular. The described antigens are shown to react with sera of patients with ABPA, but there are no data suggesting that the antigens will not react with sera of A. fumigatus-sensitised patients not having ABPA.

Moser et al (66) and Little et al., (79) describe secreted
15 proteins/antigens that do not allow for differential diagnosis of ABPA because they frequently reacts with sera of A. fumigatus-sensitised patients without ABPA and sera of ABPA patients.

20 **The objectives of the invention.**

The main objective of the invention is to provide improved methods for diagnosis of ABPA.

One subobjective is to provide in vitro diagnostic methods that have the sufficient specificity and sensitivity for
25 diagnosis of ABPA.

A second subobjective is to provide well-defined allergen preparations that can be used for the diagnosis of ABPA both in vitro and in vivo, including immunoassay and skin reactivity measurement methods, respectively.

30

The invention

The first major aspect of the invention is a method for diagnosis of allergic bronchopulmonary aspergillosis (ABPA). This aspect is characterised in using as a reagent an ABPA-
35 related recombinant allergen, i.e. a recombinant allergen carrying an epitope against which antibodies of various Ig

classes/ subclasses, such as of the IgE class or total IgG or
IgG subclasses (IgG1, IgG2, IgG3 and IgG4) can be detected so
that an ABPA condition in a patient can be differentiated
from allergic sensitization to A. fumigatus, which is
5 particularly useful in patients suffering from cystic
fibrosis.

The concept of ABPA-related recombinant allergens includes
any recombinant allergen, irrespective of origin, having the
above-mentioned antibody binding feature permitting the
10 differential diagnosis indicated. It encompasses in
particular ABPA-related recombinant allergens derived from A.
fumigatus and their ABPA-related fragments. For ABPA-related
recombinant allergens cloned from A. fumigatus, the concept
encompasses ABPA-related allergens and fragments derived from
15 other sources, having one or more ABPA epitopes in common
with an ABPA-related allergen from A. fumigatus. At the
priority, date rAsp f4 and rAsp f6 and their fragments, as
defined above, were considered to be the most useful ABPA-
related allergens. Various derivatized forms retaining the
20 ability to bind antibodies, as defined for ABPA-related
recombinant allergens, are also included.

Various subaspects include in vitro and in vivo testing
protocols as described below.

The second major aspect of the invention is novel ABPA-
25 related recombinant allergens binding to human IgE present in
ABPA patients and useful in the first aspect of the
invention.

Various subaspects of this second major aspect of the
invention are apparent from the below and encompasses
30 derivatized forms including but not limited to underivatized,
insolubilized and labelled ABPA-related allergens.

Another aspect of the invention is the use of ABPA-related
allergens for hyposensitization treatment as done for other
allergens.
35

Cloning of allergens from Aspergillus fumigatus.

The cloning strategy utilized phagemid pComb3 (47) and the
ability of the leucine zipper proteins Jun and Fos to
associate with each other (74,48,74,75).

A modified gIII product, obtained by fusing the DNA
5 encoding the jun leucine zipper flanked by cysteine residues,
N-terminal to the viral coat protein was expressed from a
LacZ promotor and secreted into the periplasmic space of E.
coli by a pelB leader peptide, thereby being structurally
incorporated into phage particles during infection with
10 helper phage (49). Using a second LacZ promotor of the
phagemid, the fos leucine zipper domain, flanked by cysteine
residues, co-expressed as N-terminal fusion peptide to cDNA
protein products of A. fumigatus, was secreted into the
periplasmic space of E. coli using the pelB leader peptide
15 (50). Through Jun-Fos heterodimerization and disulfide bond
formation, the gIII-Jun fusion protein incorporated into
phage particles provides a covalent link to phage surface for
random recombinant cDNA products with the Fos leucine zipper
attached N-terminally (48,49). The phagemid pJuFo which
20 contains the described elements (49,51,52), allows expression
and display of cDNA libraries, in this case encoding shot-gun
cloned A. fumigatus peptides/proteins, on phage surface and
application of the powerful screening technology based on
biopanning procedures used for other filamentous phage
25 systems (46,47). The key of success in cDNA cloning from
libraries displayed on phage surface lies in the screening
strategy used. The most important factor to be considered is
that the ligand used to select phage should be tagged or
immobilized in a way allowing the ligand to retain its native
30 conformation (46). It must be taken into account that
proteins, when directly immobilized to a solid phase by
hydrophobic interaction, may lose biological activity due to
alterations in the three-dimensional structure (54,55). In
general the known or expected characteristics of the ligand
35 will dictate the procedure used for ligand immobilization.
For the isolation of allergens recognized by serum

antibodies, the use of capture antibodies has proven to be very effective for different reasons. First, monoclonal antibodies raized against the immunoglobulin ε constant domains Cε2, Cε3 or Cε4 do not interfere with the antigen

5 binding site of the antibody. Second, a surface coated with such anti-IgE antibodies will be able to immobilize selectively IgE antibodies from serum of allergic patients. Therefore, after washing away interacting and cross-reacting serum antibodies of other isotypes together with all other

10 serum components, a specific surface able to adsorb only phage displaying IgE binding molecules will be obtained (51-53).

The application of pJuFo to display cDNA products and select phages from a library constructed using mRNA from A.

15 fumigatus (39,51,52) yielded a wide variety of phage clones able to bind IgE antibodies from sera of patients sensitized to A. fumigatus (table 1).

Compared with screening of λ-libraries immobilized on solid

20 phase supports, the screening procedure for cDNA libraries displayed on the surface of filamentous phage has several advantages. Capturing serum IgE with an immobilized anti-IgE antibody generates a homogenous surface with immobilized IgE which does not become denatured (56,57) and therefore retain

25 the full antigen binding capacity. The most important advantage results from the fact, that the phage library is kept in a liquid phase, where only phage with affinity to the ligand are retained on the solid phase after washing (47,53). Desorbed phage can be used to infect E. coli in order to

30 amplify phage with affinity for the ligand. Therefore, successive rounds of phage growth and selection allow enrichment of phage displaying proteins with affinity for the ligand (table 3). After selection of candidate phage clones displaying proteins with IgE binding properties, phage

35 particles produced from 10 ml culture can be precipitated (47) and samples of 1010-1013 phage particles of each

candidate clone analysed by SDS-PAGE under reducing
conditions, followed by transfer to nitrocellulose membranes
(49,51). After blocking in order to saturate free binding
sites on the nitro-cellulose sheet, membranes are incubated
5   with patient serum diluted 1:10 as "first antibody" and mouse
anti-human IgE mAb as second antibody can be used to
visualize binding of IgE to the cDNA product originally
present on the phage surface. Western blots can easily be
developed using non-radioactive systems and horse-radish-
10   peroxidase-conjugated goat-anti mouse Ig as detection system.
The apparent molecular mass of the IgE-binding proteins
enriched from an A. fumigatus cDNA library displayed on phage
surface was in the range of 10 to more than 50 kDa.
Nucleotide sequence determination (58) of some cDNA-inserts
15   differing in size and restriction pattern revealed that they
encode different proteins as deduced from the open reading
frames.

Production of recombinant allergens in E. coli.
20   Illustrative examples of production methods will now be
given for two ABPA-related recombinant allergens cloned from
A. fumigatus, designated rAsp f4 and rAsp f6.

rAsp f6: DNA encompassing the coding sequence of rAsp f6
was cloned into an expression vector under the
25   transcriptional control of the T7 promoter (78). The
construct was designed in such a way that one methionine
residue was added at N-terminal end of the allergen amino
acid sequence, while at the C-terminus the eight-residue
(SEQ ID NO/7)
stretch -VEHHHHHH_was added, of which the six consecutive
30   histidine residues serve as an affinity tag for metal-chelate
affinity chromatography (61). After sequence confirmation,
the construct was transferred to E. coli BL21[pT7POL23] (77),
in which synthesis of the T7 RNA polymerase can be induced by
raising the temperature of the growing culture to above 37°C.
35   To produce rAsp f66, 1 liter of LB medium containing an
appropriate complement of antibiotics was inoculated with 1

ml of an overnight starter culture grown at 30°C. After
approximately 3 hrs of growth at 30°C, at an OD_{600} of 0.7, the
temperature of the culture was shifted to 42°C in order to
induce expression. After 4 hrs of of incubation at inducing
5 temperature, cells were harvested by centrifugation and
resuspended in 50 ml of ice-cold 20 mM Tris-HCl pH 8.0
containing 0.5 M NaCl and 5 mM imidazol (resuspension
buffer). The cells were disrupted by sonication and insoluble
debris removed by centrifugation. The supernatant, containing
10 the overexpressed allergen, was passed through a 0.22 μm
filter to remove remaining particulate material and loaded
onto an assembly of two serially connected 5 ml HiTrap
Chelating columns (Pharmacia Biotech AB, Uppsala, Sweden)
previously charged with Ni^{2+} and equilibrated with
15 resuspension buffer. The colum assembly was washed first with
50 ml of resuspension buffer, then with 50 ml of resuspension
buffer supplemented with Imidazol to 60 mM. To elute rAsp f6,
a 30 ml linear gradient of 60-500 mM imidazol in 20 mM Tris-
HCl pH8.0/0.5 M NaCl was applied while 1 ml fractions were
20 collected and analysed by SDS-PAGE. Fractions containing rAsp
f6 were pooled and subjected to gel filtration through a
Superdex 200 column (Pharmacia Biotech AB, Uppsala, Sweden)
equilibrated and eluted with 0.15 M NaCl. Fractions
containing rAsp f6 were pooled and concentrated using an
25 Amicon cell fitted with a YM5 membrane. Final yield of
purified rAsp f6 from one liter of bacterial culture was 23
mg.

   rAsp f4: DNA encompassing the coding sequence of rAsp f4
was cloned into an expression vector under the
30 transcriptional control of the T7 promoter (78). The
construct was designed in such a way that the 11-residue
       (SEQ ID NO: 8)
β stretch MRGSHHHHHM-,was added to N-terminal end of the
allergen amino acid sequence, of which the six consecutive
histidine residues serve as an affinity tag for metal-chelate
35 affinity chromatography (61). No amino acid addition was made
at the C-terminal end of the protein. After sequence

confirmation, construct was transferred to E. coli
BL21[pT7POL23] (77), in which synthesis of the T7 RNA
polymerase can be induced by raising the temperature of the
growing culture to above 37°C. To produce rAsp f4, 1 liter of
5   LB medium containing an appropriate complement of antibiotics
was inoculated with 1 ml of an overnight starter culture
grown at 30°C. After approximately 3 hrs of growth at 30°C,
at an OD_{600} of 0.7, the temperature of the culture was shifted
to 42°C in order to induce expression. After 4 hrs of of
10 incubation at inducing temperature, cells were harvested by
centrifugation and resuspended in 50 ml of ice-cold 20 mM
Tris-HCl pH 8.0 containing 0.5 M NaCl. The cells were
disrupted by sonication and insoluble material including rAsp
f4 protein was collected by centrifugation. The insoluble
15 material was washed twice by resuspension in 20 mM Tris-HCl
pH 8.0 containing 2 M Urea, 0.5 M NaCl and 2% Triton X-100,
followed by centrifugation. Partially purified rAsp f4-
containing inclusion bodies were extracted for 45 minutes at
room temperature in 70 ml of 20 mM Tris-HCl pH8.0 containing
20 6 M guanidinium hydrochloride, 0.5 M NaCl, 5 mM Imidazol and
1 mM 2-mercaptoethanol (extraction buffer). The extract was
clarified by centrifugation and remaining particulate
material removed by passage through a 0.22 μm filter. The
clarified extract, containing the overexpressed allergen, was
25 loaded onto an assembly of two serially connected 5 ml HiTrap
Chelating columns (Pharmacia Biotech AB, Uppsala, Sweden)
previously charged with Ni^{2+} and equilibrated with extraction
buffer lacking 2-mercaptoethanol. The column assembly was
washed first with 50 ml of extraction buffer, then with 50 ml
30 of 6 M urea in 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 20 mM
Imidazol and 1 mM 2-mercaptoethanol (urea wash buffer). In
order to renature the immobilized rAsp f4, a 960 ml linear
gradient was applied, from urea wash buffer to 20 mM Tris-HCl
pH8.0 containing 0.5 M NaCl, 20 mM Imidazol and 1 mM 2-
35 mercaptoethanol (renaturation buffer). To elute rAsp f4, a 30
ml gradient of 20-1000 mM imidazol in renaturation buffer was

applied while 1 ml fractions were collected and analysed by
SDS-PAGE. Fractions containing rAsp f4 were pooled and
subjected to gel filtration through a Superdex 75 column
(Pharmacia Biotech AB, Uppsala, Sweden) equilibrated and
5   eluted with 0.15 M NaCl. Fractions containing rAsp f4 were
pooled and concentrated using an Amicon cell fitted with a
YM10 membrane. Final yield of purified rAsp f4 from one liter
of bacterial culture was 34 mg.
        Production has also been carried out with the vector
10 described by Hochli et al (60-63).

Analysis of cDNA inserts

        Only inserts coding for peptides/proteins relevant for the
diagnosis of ABPA will be discussed.
15 **rAsp f6 (SEQ ID NO 1).** A clone containing an insert of 751
base pairs with an open reading frame of 624 base pairs
revealed a strong homology with nucleotide sequences encoding
superoxide dismutases. The 3'-noncoding region had a
polyadenylated tail of 24 base pairs. The deduced amino acid
20 sequence of this cDNA clone (SEQ ID NO 1) was homologous to
manganese SOD, showing the highest sequence identity of 48-52
% to the human, fruit fly, gum tree, yeast, E. coli, and
Mycobacterium leprae enzymes. Apparently the A. fumigatus
MnSOD displays a similar high degree of sequence identiy to
25 MnSODs from a wide variety of phylogenetically distant
organisms (43). Multiple sequence alignment shows that the A.
fumigatus MnSOD (rAsp f6) shares high homology with human
MnSOD (51.8% identity, 67.2% homology). IgE raised against A.
fumigatus MnSOD is detected predominantly in sera of patients
30 suffering from ABPA. Therefore MnSOD could be a candidate for
a serologic differential discrimination between ABPA and A.
fumigatus allergy (see below). Notably, both recombinant A.
fumigatus and human MnSOD induce proliferation in peripheral
blood mononuclear cells of A. fumigatus allergic subjects
35 with detectable levels of specific IgE to A. fumigatus MnSOD.
Moreover, both the fungal and human recombinant MnSODs

elicited Type I skin reactions in individuals sensitized to the fungal enzyme, providing evidence for auto-reactivity to human MnSOD in allergic individuals sensitized to the environmental A. fumigatus allergen (43).

5   **rAsp f4 (SEQ ID NO 2).** This was the second recombinant ABPA-related allergen discovered in our screening system. The clone contained an isert of 1103 base pairs with an open reading frame of 858 base pairs. Its deduced amino acid sequence does not share significant homology to any known
10 protein. The gene product encoded by the used cDNA was only characterized by the function for which it was selected: IgE binding.

In vivo tests utilizing recombinant allergens.

15   These are mainly illustrated by skin prick tests in which a small amount of a solution of an allergen is inserted into the dermis of an individual whereupon a wheal reaction occurs around the place for administration.

    One protocol for skin prick tests of the invention implied
20 that a recombinant allergen was dissolved in 0.9% physiological saline as a diluent at an end concentration of 100 µg/ml. 20 µl of these solutions were placed on the patient's forearms. Thereafter the skin was pricked with a sterile needle, which was entered into the epidermis at a
25 degree angle and lifted up to elevate a small portion of the epidermis (38). The needle was discarded after the application of each solution to avoid contamination. The test sites were placed 3 to 4 cm apart to avoid false positive results.

30   For intradermal tests, an allergen solution (100 µg/ml) were diluted at serial 10-fold dilutions and applied at concentrations starting from 10-4 µg/ml to 10 µg/ml. For testing the solutions (100 µl) were injected on the patients' backs starting from the solution with lowest concentration
35 resulting in a size of the wheal of half the size of the skin reaction induced by the histamine control. The test sites

were placed 5 to 8 cm apart to avoid false-positive results.
Histamine dihydrochloride was used as a positive control at
concentrations of 0.1 % in skin prick tests or 0.01% in
intradermal tests, respectively. Physiological 0.9% salime

5 was used as a negative control. The reactions were recorded
after 15 minutes by measuring the maximal longitudal and
transversal diameter of the wheal and evaluated as described
(66).

10

The use of recombinant A. fumigatus allergens for in vitro diagnostics.

The binding of recombinant A. fumigatus allergens to
antibodies may be used in immunoassays for measuring

15 allergen/antigen specific antibodies of various classes (IgA,
IgG, IgD, IgE and IgM), including specific subclasses
thereof, for instance in connection with diagnoses of allergy
and ABPA. Among IgG subclasses may be mentioned IgG1, IgG2,
IgG3 and IgG4. The methodology for the assays is the same as

20 that used in the prior art for conventional
antigens/allergens. Suitable immunoassay protocols thus
contemplate formation of a ternary immune complex:

[allergen] - [anti-allergen antibody] - [anti-antibody]
where allergen and anti-antibody are added reagents and anti-

25 allergen antibody derives from the sample to be assayed. The
complex is formed in an insoluble or insolubilizable form.
Insoluble forms are accomplished by having either the
allergen or the anti-antibody bound to a solid phase before,
after or during formation of the complex. Well known solid

30 phases in the field are walls of tubes and wells, particulate
and monolithic more or less porous materials used as
adsorbents in chromatography and heterogeneous imunoassays
etc. In order to measure the amount of complex, either the
allergen or the anti-antibody is labelled with an

35 analytically detectable group, with the provision that the
reagent linked to a solid phase or causing post-

insolubilization is not labelled. Well known detectable groups are enzymes (ELISA), fluorophors, chromophors, chemiluminescent groups, radioactive isotopes, metal atoms, biotin, haptens etc. In order to measure class/subclass

5 specific antigen/allergen specific antibodies the anti-antibody has to be class/subclass specific.

Normally this type of immunoassay is run with sequential incubation, i.e.

step 1: sample with allergen followed by

10   step 2: incubation of the complex formed in step 1, i.e. [allergen] - [anti-allergen antibody] with anti-antibody

or vice versa. In case the reagent used in step 1 is bound to a solid phase, separation and washing after each step should

15 be carried out in order to remove unspecific interference.

For ABPA diagnosis, IgE and certain IgG subclasses are the most relevant Igs to measure. It is believed that the recombinant allergens to be used should be derived from A fumigatus proteins not being exposed on the cell surface or

20 secreted. This may indicate that the most relevant A. fumigatus allergens relevant for ABPA may be cell-bound, for instance as intracellular peptides/proteins.

Relevant antibodies can be found in blood (including plasma and serum), saliva, cerebrospinal fluid (CSF),

25 bronchioalveolar fluid, tear drops (lacrymal fluid) etc.

The in vitro test protocols used and results.

The binding of IgE antibodies (and other isotypes) to recombinant allergens was assessed by an ELISA (39) using the

30 same method for all allergens. Briefly, polystyrene microtiter plates were coated for 2 h at 37°C with allergen protein (10 μg/ml in PBS, pH 8.0). The free sites were blocked with PBS, pH 7.4 containing 5 % (w/v) non-fat dry milk powder (1 h, 37°C). After washing, the plates were

35 incubated with serially twofold-diluted sera in blocking buffer containing 5% Tween 20 (2 h, 37°C). After washing, a

second antibody of commercial source (66) or TN-142, a mouse monoclonal anti-human IgE antibody raised against the Cε2 domain (kindly supplied by Dr C.H. Heusser, Ciba-Geigy Ltd., Basel. Switzerland) were used to quantify the isotype-
5 specific Ig-content of the sera. Isotype-specific Ig-binding to the allergens was detected with alkaline-phosphatase-conjugated goat anti-mouse IgG (66, 69). In absence of calibrated standards, a serum pool from two patients suffering from ABPA was used as an in house reference. Serum
10 dilutions versus optical density were plotted in a log-log diagram and the linear titrable region used to convert the optical density values to arbitrary ELISA units (EU). Absorbence values from the reference serum pool were arbitrarily set as 100 EU/ml for all isotypes analysed
15 (66,68). The antigen-specific ELISA allows reliable detection of serum antibodies. For the IgE-determinations using rAsp f6, the results have been validated using Pharmacia CAP System (Pharmacia & Upjohn, Diagnostics, Uppsala, Sweden) with the recombinant proteins as immobilized allergen.
20 For a large scale evaluation of the in vitro diagnostic value of recombinant A. fumigatus allergens, 54 sera from patients suffering from ABPA and from 35 allergic asthmatics with A. fumigatus sensitization but without ABPA as deduced from the clinical parameters were selected. All patients had
25 asthma and met the guidelines for the diagnosis and management of asthma (70). As negative control, sera from 10 allergic asthmatics without A. fumigatus sensitization and from 10 healthy individuals without history of atopy were used. In contrast to sera from sensitized individuals, the
30 serum samples of the 20 control individuals showed IgE values below the background for all recombinant allergens, demonstrating that the IgE detection system is related to specific sensitization to A. fumigatus. The results of the IgE determinations obtained with sera of A. fumigatus
35 allergic asthmatics with or without ABPA for the relevant

recombinant allergens so far discovered (rAsp f4 and rAsp f6) will be discussed below.

The serological investigations rAsp f4 and rAsp f6 show a completely different picture compared to that obtained with

5 other recombinant A. fumigatus allergens. Specific IgE against rAsp f4 and rAsp f6 was not detectable in the 35 sera from allergic asthmatics sensitized to the fungus. In contrast, the 54 sera from ABPA-patients recognized rAsp f4 and rAsp f6 at a frequency of 54% and 78%, respectively,

10 (table 3), whereas 49 sera recognized at least one of the allergens. Therefore, serologic diagnosis of ABPA with the two allergens has a specificity of 100 % and a sensitivity > 90% (table 4). The MnSOD (rAsp f6), a protein with a known biochemical function, represents a strictly intracellular

15 enzyme. The biobiological function of Asp f4 remains unknown; however, preliminary experiments to locate the protein using monoclonal antibodies raised against Asp f4 indicate that the protein is not secreted by the fungus. Therefore both proteins are unlikely to be present in free form as

20 aeroallergens, which may explain the lack of specific IgE against these allergens in allergic asthmatics sensitized to A. fumigatus. In contrast, patients suffering from ABPA have or have had the fungus growing in the lung (8,12) and as a result of disintegration of fungal cells by host defence

25 mechanisms, become exposed also to non-secreted proteins (3). One of the host defence mechanisms against fungal infections consists of the damage of hyphae and phagocytosis mediated by polymorphonuclear cells (2,3,4). Development of a cell-mediated immune response to a fungus is thought to require

30 antigen-presenting cells to process and present fungal antigens to T-lymphocytes (1). Therefore patients suffering from ABPA are able to mount an immune response also to intracellular proteins of A. fumigatus never seen by the immune system of A. fumigatus-allergic individuals, which are

35 exposed only to secreted allergens and conidiae. The in vivo relevance of rAsp f4 and rAsp f6 has been assessed in skin

tests involving representative numbers of patients with ABPA, A. fumigatus allergy and healthy controls (see below).

Diagnostic value of recombinant A. fumigatus allergens for in vivo tests.

Regarding a potential discrimination between ABPA and allergic sensitization, the most significant findings of the serologic investigations, involving subjects with asthma and concomitant sensitization to A. fumigatus were elevated levels of specific serum IgE to rAsp f4 and rAsp f6 in patients suffering from ABPA. As indicated in table 3, rAsp f4- and rAsp f6-specific IgE, as measured by ELISA, reached values of 54±160 ELISA Units/ml and 47±66 ELISA Units/ml in sera of asthmatic patients with ABPA. In contrast, specific IgE antibodies to these two allergens were virtually absent in sera of asthmatic patients sensitized to A. fumigatus without evidence for ABPA, as well as in sera of control individuals (table 3). Based on these results, rAsp f4 and rAsp f6 could serve as reagents for the development of an ABPA-specific assay based on circulating allergen-specific IgE antibodies. It was therefore of interest to assess the allergenicity of these proteins in vivo. To demonstrate the ability of rAsp f4 and rAsp f6 to elicit mediator release in vivo, an intradermal skin provocation study was carried out involving 12 asthmatic patients with ABPA, 12 allergic asthmatics sensitized to A. fumigatus without ABPA and 5 healthy controls. Selection of patients and diagnosis of sensitization to A. fumigatus were based on clinical history, RAST and skin reactivity to A. fumigatus extracts as described (59,66). All patients had asthma and met the guidelines for the diagnosis and management of asthma (70). At the time of the study all subjects had stable bronchial asthma, no evidence for chest infections and received no anti-histamine medication. The five healthy control individuals had no history of allergy or asthma and had normal serum levels of total IgE. The diagnosis of ABPA was

based on a minimum of six of the eight criteria proposed by
Rosenberg et al (23) and Patterson et al (24). Four ABPA
patients (table 5) and one patient with allergic asthma
(table 5) were treated with low doses of oral corticosteroids
5 (5-10 mg/day). An ethical approval for skin testing human
subjects with recombinant allergens was obtained from the
responsible committee before starting the study. A full explanation
of the procedure was given to all individuals before testing
and subsequently a written consent was obtained. The main
10 characteristics of the subjects participating in the study
including age, sex, eosinophil count, total serum IgE,
specific serum IgE to rAsp f4 and rAsp f6 and RAST to A.
fumigatus are reported in table 5. All subjects showed a
positive skin test response to intradermal histamine
15 challenges (0.01%) and were non-reactive to 0.9% saline. The
results (table 5) suggest a high specificity of rAsp f4 and
rAsp f6 reactivity for patients suffering from ABPA. In fact,
only this group of patients showed relevant amounts of
specific IgE against rAsp f4 and rAsp f6 (Table 3 and 4). As
20 expected, only individuals showing detectable amounts of
allergen-specific IgE in serum reacted to skin challenges
with rAsp f4 and rAsp f6. These results clearly show that a
highly specific diagnosis of ABPA based on recombinant
allergens is feasible. However, although rAsp f4- and rAsp
25 f6- based serology and skin tests show a high specificity for
ABPA in the absence of atopic dermatitis, the sensitivity of
the diagnosis reaches only about 90% (table 4). Taking into
account the the relatively low specificity of the diagnostic
criteria for ABPA available to date, serological and skin
30 tests with rAsp f4 and rAsp f6 represent a considerable
improvement of the diagnosis of the disease. Moreover, the
characteristics of both allergens, taken together with the
observation that ABPA patients and A. fumigatus sensitized
allergic asthmatics recognize different allergen in Western
35 blot analysis, provide a rational for a further improvement
of the diagnosis of ABPA. In a study reported by Borga (6),

serum IgE-reactivity to A. fumigatus allergens in two groups
of patients were compared, A. fumigatus sensitized allergics
and ABPA patients. Sera of individuals suffering from A.
fumigatus allergy recognized at least thirty-five different
5   IgE-binding components of the fungus, ranging between 14 and
118 kDa in size, of which four components (34, 39, 43 and 83
kDa) were uniquely detected by these sera. With sera from the
ABPA group, thirty-nine different IgE-binding components
ranging from 14 to 150 kDa were detected, of which eight
10 components with molecular weights of 15, 19.5, 54, 56, 96,
110, 126 and 150 kDa were not recognized by IgE from the
allergics. Therefore, from the total of 43 IgE-binding
components detected, antibodies to 31 were found in both of
the patient groups, 8 were specific for ABPA and 4 specific
15 for non-ABPA-related sensitization to A. fumigatus.

The availability of the recombinant allergens described
will allow identification of A. fumigatus-allergic who lack
sensitization to these cloned allergens. Sera from such
subjects can subsequently be used to screen the A. fumigatus
20 phage surface display library in order to isolate phage
clones displaying additional allergens. The powerful
screening procedure based on biopanning (45,47,51,52),
together with a rational for the selection of the sera used
to screen the phage library, will allow isolation of the
25 additional allergens in a reasonable time. Production,
characterization and evaluation of these allergens are likely
to contribute to the further development specific diagnosic
tools for both ABPA and A. fumigatus-related sensitization.

In order to use rAsp f6 as a specific allergen for the
30 diagnosis of ABPA, atopic dermatitis has to be excluded. A
high percentage of patients suffering from atopic dermatitis
with a moderate RAST class to A. fumigatus shows high titres
of rAsp f6-specific IgE in serum. Moreover, intradermal skin
challenges with rAsp f66 in three patients suffering from
35 atopic dermatitis clearly demonstrated that the allergen is
able to provoke a strong in vivo mediator release in these

patients. Notably the serologic investigation of 15 sera of patients suffering from atopic dermatitis does not show any specific IgE to the other recombinant A. fumigatus allergens available (76). The reason for the monovalent sensitization

5 to Asp f2 in patients with atopic dermatitis is unknown. However, it is tempting to speculate that the specific IgE response against rAsp f6 could be due to production of IgE antibodies recognizing human superoxide dismutase in these individuals, which would result in a cross-reaction to the

10 highly homologous fungal MnSOD (43). The availability of both the human and fungal recombinant MnSOD will allow a study of the role of these proteins in the pathophysiology of atopic dermatitis in more detail.

15

Serologic discrimination between sensitization to A. fumigatus and ABPA in patients with cystic fibrosis.

This study involved 37 patients with cystic fibrosis with routine assessment for cystic fibrosis and allergy, including

20 skin prick testing (67,71). 15 were diagnosed as having ABPA according to the clinical and immunological criteria proposed by Laufer (16) and Nelson (25). 12 patients belonged to the group with documented sensitization to A. fumigatus according to RAST and routine skin prick test to A. fumigatus extracts

25 and 10 were assigned to the CF control group based on the lack of sensitization. to A. fumigatus (67). Patient characteristics including age, sex, RAST to A. fumigatus and total serum IgE are reported in table 6. Allergen specific IgE levels were determined for the allergens rAsp f1, rAsp

30 f3, rAsp f4 and rAsp f6 in serum of each individual. rAsp f1 (43) and rAsp f3 (42) correspond to major allergens of A. fumigatus with a prevalence of sensitization of 69% and 76% among asthmatic patients with positive skin test to A. fumigatus extracts, whereas rAsp f4 snd rAsp f6 (43) are

35 recognized only by sera of patients with ABPA. All four proteins have been demonstrated to be relevant allergens in

vivo by skin challenges of asthmatic patients sensitized to
A. fumigatus. The results of the serological investigation
(table 9) show that the majority of the cystic fibrosis
individuals sensitized to A. fumigatus are carry IgE to rAsp

5   f1 and rAsp f3 (85% and 100%, respectively) and 85% to both
allergens. Taken together rAsp f1 and rAsp f3 are sufficient
to diagnose sensitzation to A. fumigatus in all of the
investigated sera from cystic fibrosis patients. According to
the current definition of allergens (41) rAsp f1 and rAsp f3

10 correspond to major allergens also for the group of cystic
fibrosis. In the three subgroups of individuals analyzed,
cystic fibrosis patients with A. fumigatus sensitization,
with or without ABPA, and cystic fibrosis individuals without
A. fumigatus sensitization, relevant levels of serum IgE

15 against rAsp f4 and rAsp f6 were found only in sera of
individuals with a clinical diagnosis of ABPA. In this group
rAsp f6-specific IgE levels exceeding the cut off value (> 5
U/ml) were detected in 10 of 15 patient sera, while relevant
levels of rAsp f4-specific IgE (cut off value > 7 EU/ml) were

20 detected in 13 of the 15 patient sera. If we consider
elevated levels of either rAsp f4- or rAsp f6- specific IgE
sufficient to indicate ABPA, all patients were covered by the
serological diagnosis, whereas 8 patients of 15 had elevated
levels of IgE to both rAsp f6 and rAsp f4. Therefore

25 allergen-specific serology with rAsp f4 and rAsp f6 could
give a substantial contribution to a differential serological
diagnosis of ABPA in patients suffering from cystic fibrosis
and A. fumigatus sensitization.

Table 1. Typical enrichment of phage from a cDNA phage display library by biopanning. Phage displaying cDNA-products from an A. fumigatus expression library were applied to a single well of a microtitre plate coated with human serum IgE (51,52). After adsorption and extensive washing adherent phages were eluted and used to infect E. coli for a further round of phage growth and selection.

Round of panning	Phage input[a]	Phage output[b]	Enrichment factor[c]	IgE-specific phage phage tested[d]
1	1.8×10^{11}	4.5×10^3	5.6×10^{-6}	0/10
2	8.6×10^{10}	2.4×10^4	5.6×10^{-5}	0/10
3	9.3×10^{10}	4.6×10^4	3.8×10^{-5}	0/10
4	6.5×10^{10}	1.1×10^5	1.9×10^{-4}	1/10
5	1.8×10^{11}	3.8×10^6	2.3×10^{-3}	8/10
6	2.1×10^{11}	8.4×10^6	4.0×10^{-3}	10/10
7	9.4×10^{10}	5.8×10^7	5.6×10^{-2}	10/10

a) Number of phage applied to a single well of a microtitre plate.

b) number of phage eluted from the well after washing.

c) Percentage yield of phage from each round of panning (%yield = [No. of phage eluted x 100]/[No. of phage applied]).

d) Single colonies from plates used to titrate phagemids were grown in liquid culture, phage induced and purified. Phage coated directly to microtitre plates were tested for IgE binding capacity by an IgE-specific ELISA (66). IgE-specific phage represents phage able to bind serum IgE/number of phage tested.

Table 2. Main characteristics of phages isolated from an A. fumigatus cDNA library displayed on a phage surface and subjected to selective enrichment using patient serum IgE as ligand.

Phage No	Insert[a] length bp	Mw of the[b] protein(kDa)	Expression[c] mg/1 culture	IgE-binding[d] frequency	Preliminary[e] designation	Skin Test[f] reactivity
2	1103	40	36	<50%	rAsp f4	+
7	854	10	65	<50%	rAsp f7	nt
19	751	28	220	<50%	rAsp f6	+
28	616	21	38	<50%	rAsp f3	+
38	1123	33	25	<50%	rAsp f9	nt
46	686	18	140	<50%	rAsp f1/a[g]	+
48	1270	42	45	<50%	rAsp f5	+
51	978	34	29	<50%	rAsp f10	nt

a) Determined from the nucleotide sequences

b) Estimated from polyacrylamide gels

c) Produced as inclusion body protein after subcloning of the fragments into pDS76/RBSII, 6xHis (62). The yields represent mg of Ni2+-chelate affinity purified proteins per litre culture (60,61).

d) Sera from 54 patients with ABPA and from 35 individuals sensitized to A. fumigatus were tested for the presence of specific IgE to the recombinant proteins. IgE-binding frequency was assigned to major (>50%) or minor (<50%) allergens (41).

e) Nomenclature not officialized.

f) Skin test reactivity determined as described (66), nt = not tested.

g) This allergen has been previously described.

Table 3. Sensitization of asthmatic patients with ABPA or A.
fumigatus allergy to recombinant allergens rAsp f4 and
rAsp f6.

Subject groups	rAsp f6	rAsp f4
ABPA (n=54)	54±160a	47±66
sensitized	30(56%)b	42(78%)
Allergic (n=35)	1±1	2±3
sensitized	0(0%)	0(0%)
ABPA+allergics (n=89)	33±89	29±56
sensitizedb	30(56%)	42(78%)
Healthy controls (n=20)	<5	<5
sensitized	0(0%)	0(0%)

a. Mean value of IgE-binding to the allergen + SD (ELISA
Units/ml).
b. Number and % of samples showing IgE s sensitized to the
allergen above cut-off level (5 and 7 EU/ml for rAsp f4
and rAsp f4, respectively).

Table 4. Discrimination between ABPA and sensitization to A.
fumigatus by rAsp f4 and rAsp f6 IgE-specific
serology.

| Subjects | Number (%) individuals sensitized to allergen | | | |
	rAsp f6	rAsp f4	rAsp f6/rAsp f4	rAsp f6+rAsp f4
ABPA (n=54)	30(56%)	42(78%)	49(91%)	25(46%)
A.fumigatus allergics (n=35)	0(0%)	0(0%)	0(0%)	0(0%)
Controls (n=20)	0(0%)	0(0%)	0(0%)	0(0%)

Specificity and sensitivity for recognition of sera from
ABPA-patients

Specificity	100%	100%	100%	100%
Sensitivity	56%	78%	91%	47%

Table 5. Principal characteristics of the subjects studied and skin reactivity to rAsp f4 and rAsp f6.

Subject	age y	sex	Eos/ml x106	Total IgE kU/l	RAST	Specific IgE toa rAsp f6	rAsp f4	Skin test tob rAsp f6	rAsp f4
ABPA									
1	62	f	0.71	475	3	22	0	++	-
2	46	m	0.37	1508	4	177	48	++++	+++
3	28	m	0.45	4576	5	346	18	+++	+
4	59	f	0.35	10957	5	1	537	-	++
5	56	m	0.91	637	5	0	11	-	++
6	52	m	0.19	2476	5	45	46	++++	++
7	55	f	-0.24	1779	5	86	33	++++	++
8	32	m	0.57	1472	4	0	82	-	++++
9	53	m	0.23	ndc	nd	1	1	-	-
10	62	m	0.15	nd	nd	0	18	-	+
11	60	f	0.08	629	4	0	47	-	+++
12	43	m	0.53			29	8	+++	++
A. fumigatus allergy									
1	32	m	0.08	4913	1	4	1	-	-
2	60	m	0.20	76	3	0	0	-	-
3	30	f	0.52	67	3	1	2	-	-
4	43	f	0.05	3328	5	0	0	-	-
5	49	f	0.75	354	3	2	0	-	-

Subject	age y	sex	Eos/ml x106	Total IgE kU/l	RAST	Specific IgE to a) rAsp f6	rAsp f4	Skin test to b) rAsp f6	rAsp f4
6	34	m	0.19	354	2	0	0	-	-
7	46	f	0.68	494	3	0	1	-	-
8	59	m	0.53	116	nd	1	2	-	-
9	58	m	0.84	>2000	3	1	1	-	-
10	57	m	0.05	nd	nd	0	1	-	-
11	35	f	0.41	>2000	4	0	0	-	-
12	44	f	0.26	1759	2	1	2	-	-
Healthy controls									
1	44	m	0.18	148	0	0	0	-	-
2	33	m	0.40	25	0	1	0	-	-
3	30	f	0.16	39	0	0	0	-	-
4	34	m	0.43	18	0	0	0	-	-
5	41	m	0.26	61	0	0	1	-	-

a) Relative Elisa Units/ml

b) Positive reaction to intradermal skin challenges with rAsp f6 and rAsp f4 at concentrations of 1 μg (+), 100 ng (++), 10 ng (+++), or 1 ng (+++).

c) nd 0 not determined.

Table 6. Serologic investigations of patients with cystic fibrosis with or without sensitization to A. fumigatus extracts.

Subject	age y	sex	Total IgE kU/l	RAST	Specific IgE to rAspa			
					f1	f2	f3	f4
1	11	f	2398	4	1224	1	265	76
2	12	f	5226	4	233	5	867	35
3	7	m	3804	4	747	733	4027	405
4	17	m	5423	4	173	100	658	53
5	22	f	1532	4	65	5	502	44
6	15	f	1017	4	45	30	178	13
7	16	m	ndb	4	1439	381	749	422
8	28	m	2126	4	122	23	340	17
9	12	m	866	nd	1037	60	3511	524
10	16	m	945	4	402	5	1057	27
11	28	m	576	4	208	346	287	18
12	19	m	928	3	48	97	183	4
13	16	m	nd	3	195	55	145	29
14	14	m	nd	nd	81	3	128	43
15	27	f	491	4	115	118	1529	3
A. fumigatus allergy								
1	12	m	435	4	63	5	453	2
2	30	f	554	4	45	5	21	2
3	24	f	323	4	2	1	1176	3
5	24	m	166	3	5	0	32	3

Subject	age y	sex	Total IgE kU/l	RAST	f1	f2	f3	f4
6	14	f	146	3	7	3	117	1
7	21	m	304	4	145	1	121	4
8	33	m	372	3	91	0	437	3
9	16	f	103	3	3	0	131	3
10	29	f	270	3	21	0	88	2
11	28	m	47	2	87	2	124	4
12	31	m	409	3	167	2	128	0
Cystic fibrosis controls								
1	20	f	56	0	2	2	9	5
2	30	m	115	0	3	4	7	2
3	30	m	nd	0	5	0	3	2
4	26	f	nd	0	1	0	1	0
5	31	m	40	0	4	0	3	2
6	32	m	59	0	6	2	4	3
7	30	m	22	0	5	1	3	2
8	36	f	nd	0	4	1	3	0
9	24	f	nd	0	1	0	4	3
10	33	m	27	0	2	0	1	0

Specific IgE to rAspa

a) Relative ELISA Units/ml

b) nd = not determined

Results obtained during the priority year.

The cDNA encoding rAsp f8 was isolated and expressed in the same way as rAsp f4 and rAsp f6 and corresponds to the coding sequence for a P_2 acidic ribosomal protein and represents

5 therefore a classical non-secreted protein. Although not tested clinically, the protein represents an IgE-binding protein when evaluated in ELISA according to the procedures given above. All results so far obtained from ELISA show that rAsp f8 is highly specific for sera of patients suffering

10 from ABPA. None of the 35 allergic asthmatics tested showed detectable levels of rAsp f8-specific IgE (2.3 ± 0.4 EU/ml) which is not statistically different from the value obtained for the 20 healthy individuals (1.2 ± 0.6 EU/ml). In contrast, 17 of the 54 patients suffering from ABPA (31 %)

15 were clearly sensitized to rAsp f8. The mean EU/ml value of the whole sample corresponds to 8 ± 14 of the overalls sensitization involving all A. fumigatus-sensitized patients (allergics + ABPA, n = 89) corresponds to 19 % (EU/ml 5.4 ± 12). However. This new ABPA-specific allergen do not

20 contribute to the improvement of the differential diagnosis of ABPA because all patients recognizing rAsp f8 already recognize rAsp f4, rAsp f6 or both of them.

The DNA sequence for rAsp f8 is shown as SEQ ID NO 5 and the corresponding amino acid sequence as SEQ ID NO 6. The

25 sequences are prelimary determined and may be incorrect at up to 10 positions, e.g. positions 92, 94, 108, 156 and 183 in the DNA sequence for rAsp f8 have not been finally confirmed.

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